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13. ABSTRACT (Maximum 200 Words)

Serine-dependent carboxylesterases (E.C.3.1.1.1) are found in a variety of tissues with high activity detected in the human liver. Carboxylesterases (CaE) hydrolyze aliphatic and aromatic esters, and aromatic amides; and play an important role in the detoxification of xenobiotic chemicals that contain organophosphate (OP) compounds. Thus, an injectable form of human hCaE should prove to be a valuable antidote for protecting soldiers from these chemical agents. The goals of this project are to over express a functional human liver hCaE from a recombinant cDNA in a human cell line, and isolate and purify the recombinant protein. To accomplish these goals, the cDNA encoding hCaE was altered in order to convert it to a secretory form. Expression of the site-mutated cDNA in cell culture resulted in the secretion of an active hCaE into the growth medium. Thus, the secreted hCaE enzyme will be concentrated and purified using hydrophobic interaction chromatography, Cibacron blue affinity chromatography, and isoelectric focusing chromatography. The long-term goal of the project is to isolate quantities sufficient for crystallization with organophosphate agents and to evaluate its use for protection by enzymatic detoxification of organophosphate nerve agents in an animal model.

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504 Scott Street
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Re: annual progress report: DAMD17-00-1-0518 - Expression and Purification of a Potential Antidote for Organophosphate Warfare Agents

Kenneth D. Lanclos, Ph.D., Principal Investigator

Introduction:

The serine-dependent carboxylesterases (E.C. 3.1.1.1) are found in many different species (1-8), and in a variety of tissues (9-14), with high activities detected in the liver. Generally, carboxylesterases exist as 60 kDa monomers, but a few associate to form homotrimers of approximately 180 kDa (1-4). cDNA clones have been obtained by screening lambda gt11 expression libraries. Their screenings have resulted in the expression of five isoenzymes of rat liver carboxylesterase (5-9) and at least two isoenzymes from the human liver (10-12). The cDNA sequences are, generally, 1.7 to 1.9 kb in size, and they encode mature proteins that range from 507 to 568 amino acids. The different isoenzymes of liver carboxylesterases are all N-linked glycoproteins of the high mannose type (5,6,12,13). Core glycosylation of the carboxylesterases occurs in the endoplasmic reticulum lumen (14,15), and, thus it is necessary to stabilize the active conformation of the protein (6,16). Whereas, most glycosylated proteins are secretory, the carboxylesterases are localized to the luminal side of the endoplasmic reticulum, especially in humans (4-6). Very small amounts of liver carboxylesterase, however, are present in the serum (17). Carboxylesterases can hydrolyze a variety of substrates in the serum, including aliphatic and aromatic esters, and aromatic amides (18). An important function of these enzymes may be the hydrolysis, and subsequent detoxification, of pesticides, insecticides and drugs (18), many of which contain organophosphate compounds that bind covalently to the active site of the enzymes (19,20). In this regard, the carboxylesterases generally function as a high affinity-low capacity detoxification mechanism, in which organophosphates react in an irreversible 1:1 stoichiometry (17). Thus, the detoxifying ability of carboxylesterase is limited by its low concentration in serum where it encounters organophosphate compounds. A treatment for organophosphate toxicity is the administration of oximes, which reactivate inhibited acetylcholinesterase and restore cholinergic neurotransmission (17). Species with high levels of serum carboxylesterase, such as rats and mice, achieve a higher level of oxime-induced reactivation of organophosphate-inhibited acetylcholinesterase than species with lower levels of carboxylesterase; this suggests that oximes also reactivate organophosphate-inhibited carboxylesterase (17). This recycling of organophosphate-

inhibited carboxylesterase provides additional protection by making the enzyme available for further binding to organophosphates, and thus, increased detoxification.

Body:

The goals of this project are to over express a functional human liver hCaE from a recombinant cDNA in a human cell line, and isolate and purify the recombinant protein. To accomplish these goals, the cDNA encoding hCaE was altered in order to convert it to a secretory form. Expression of the site-mutated cDNA in cell culture resulted in the secretion of an active hCaE into the growth medium. Thus, the secreted hCaE enzyme will be concentrated and purified using hydrophobic interaction chromatography, Cibacron blue affinity chromatography, and preparative isoelectric focusing chromatography.

The long-term objective of the project is to isolate quantities sufficient to evaluate its use for protection by enzymatic detoxification of organophosphate nerve agents in an animal model. The short-term goals of this study are to maximize the expression of a functional recombinant secretory form of human liver carboxylesterase using a steady state human cell culture system, and to isolate and purify the recombinant enzyme from the culture media.

Key Research Accomplishments: (July 1, 2002 to July 26, 2003)

Stable clones of 293T cells, stably transfected with the plasmid pRC/mhCaE were placed in liquid culture for expression of the secretory form of human liver carboxylesterase. Carboxylesterase activity was observed to increase as early as 24 hours and to reach a maximum at day four (21). Cultured cells were separated from the media by low speed centrifugation and the pooled media from several cultures was used for the isolation of purification enzymatically active enzyme.

Carboxyleasterase was precipitated from the pooled media and precipitated using 70% saturated ammonium sulfate. The precipitate from the ammonium sulfate fraction was dissolved in 20 mM Hepes, pH 7.0 and applied to an octyl Sepharose column (21) for Hydophobic Interaction Chromatography (HIC). Previous observations had shown that HIC chromatography using octyl Sepharose, coupled with octyglucophranoside in the elution buffer resulted in an enhanced partial purification of carboyxlesterase. However, the major contaminant of the preparation, bovine serum abumin, as well as several minor contaminants, were not removed by this procedure.

Based on our previous observations (21) that carboxylesterase binds to many matrices used in standard protein purification procedures, we developed a liquid isoelectric focusing preparative system. The strategy was to remove the bulk of the BSA

(pI 4.9) from the carboxylesterase (pI 5.3) by precipitation of the BSA into the bottom of the column at around pH 4.0. A typical isoelectric focusing chromatogram (Figure 1) shows that BSA spills over into the pI 5.3 region and contaminates the carboxlesterase peak at pI 5.3.. The fractions containing carboxylesterase activity and the small amount of contaminating BSA were then collected and used for a second run on the isoelectric focusing column.. Following the second isoelectric focusing, the contaminating media proteins completely separate from the carboxylesterase fractions (figure 2). Furthermore, polyacrylamide gel electrophoresis zymograms of the carboxylesterase peak show only a single carboxylesterase band.

One of the major goals of this project was to be able to purify sufficient carboxylesterase to allow for crystallization studies on the enzyme. To date, we have provided 6mg of pure recombinant human liver carboxylesterase to DR. Steven Kirby, USAMRICD to begin a collaboration crystallization studies with OP compounds.

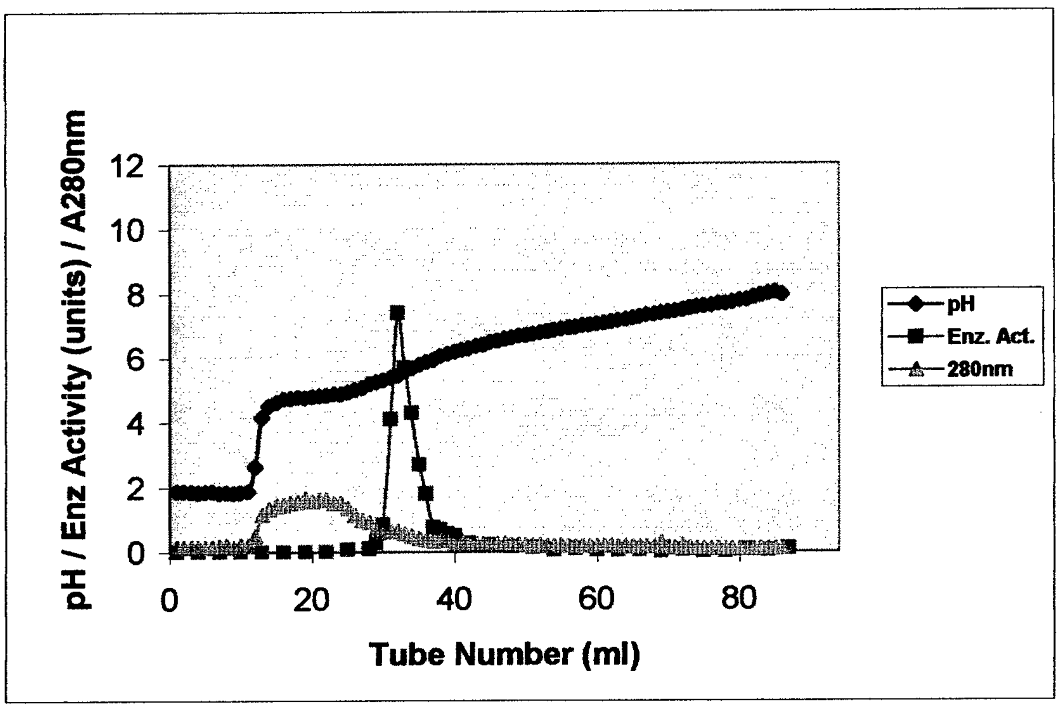

Figure 1: Preparative Electric Focusing Chromatogram

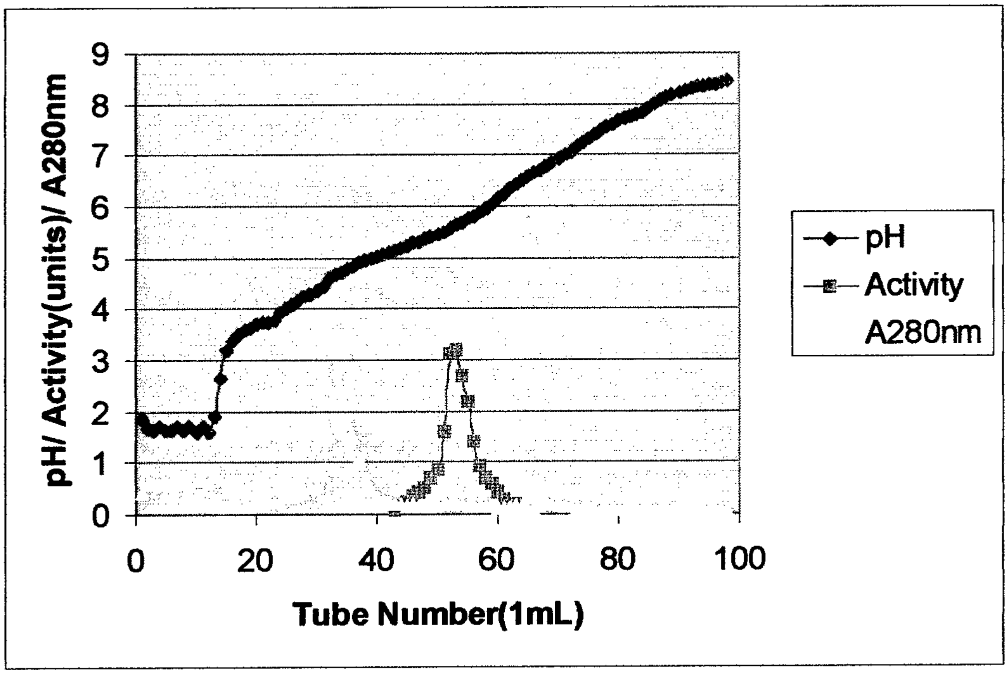

Figure 2: Preparative Electric Focusing Chromatogram

Conclusion:

Clones containing the secretory form of human liver carboxylesterase were used to establish culture conditions where the enzyme is produced maximally in the 293T human cell line. This objective was accomplished by growing cells in a chemostat where the cells are maintained in a constant exponential phase of growth. In addition, conditions were defined which allow for an optimal time of removal of the culture media for isolation of carboxylesterase. Enzyme was isolated from the culture media, and the initial steps of enzyme purification using ammonium sulfate precipitation, HIC chromatography, and preparative liquid isoelectric focusing were accomplished. In the final steps of purification, the use of preparative liquid isoelectric focusing chromatography was found to be productive and resulted in a preparation that was free of bovine serum albumin and other minor contaminants as measure by polyacrylamide gel electrophoresis. Pure carboyxlesterase has been supplied to the USAMRICD to begin crystallization studes with OP compounds.

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